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Changes in Concentration of Botulinum Toxin in Dog Serum After Parenteral Administration

by
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September 1964

Edgewood Arsenal, Maryland 21010

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FOREWORD

This work was authorized under Project 1C622401A097, Medical Defense Aspects of Chemical Agents (U). Data are recorded in notebooks MN-1703 and MN-1721. The laboratory work was started in February 1963 and completed in June 1963

In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

Notice

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DIGEST

Botulinum toxin, type A, in doses of 8,000 and 10,000 MU/kg, was administered to dogs by the intravenous (iv), intraperitoneal (ip), and intramuscular (im) routes. (MU = mouse unit, or mouse ip LD50.) Blood samples were taken at various times after injection, and the concentration of toxin in the serum was determined by a bioassay in mice, using a quantal (mortality fraction) or a graded (time to death) response. The percent of the injected dose found in the serum was plotted against sampling time to show the extent of transfer of toxin into the bloodstream from the ip and im routes and its disappearance from the blood.

It was concluded that:

1. When botulinum toxin is administered ip and im to dogs, peak serum levels are reached at 12 hr (ip = 12%; im = 9%). At 22 hr, blood levels are identical (8.5%) by the iv, im, and ip routes and remain identical, although decreasing, for many hours thereafter.

2. Botulinum toxin administered iv, ip, and im in doses of 10,000 MU/kg to dogs can be detected in the serum by bioassay in mice for as long as 2 to 4 days after injection.

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CHANGES IN CONCENTRATION OF BOTULINUM TOXIN IN DOG SERUM AFTER PARENTERAL ADMINISTRATION

I. INTRODUCTION.

This experiment was planned to determine the amount of free botulinum toxin, type A, in blood of dogs at various times after parenteral administration. Since this toxin is a protein molecule with a molecular weight of approximately 900,000, the toxin would probably be slowly absorbed into the blood after its intramuscular (im) or intraperitoneal (ip) injection. The extent of its transfer into the bloodstream and its eventual disappearance from the blood when given by these two routes was compared with that of toxin given intravenously (iv).

II. MATERIALS.

Mongrel dogs weighing approximately 10 kg were used. Mice weighing 25 gm were used as the assay animals.

The botulinum toxin, type A, was similar to that described by Lamanna, McElroy, and Eklund.[1] The mouse ip LD50 (mouse unit, MU) for this partially purified toxin was approximately 3×10^{-4} μg, which is about 10% of that for the crystalline product. The stock solution was prepared by personnel of the Basic Toxicology Branch from the powdered material dissolved in a sterile gelatin-phosphate buffer solution (10 gm of Na_2HPO_4 and 2 gm of Difco gelatin in 1 ℓ of distilled water); the pH was adjusted to 6.8 by addition of concentrated HCl. All stock solutions were bioassayed in mice for potency before they were used. The potency of the injected solutions was 20,000 MU/ml.

Bivalent botulinum antitoxin (equine origin), a globulin-modified, types A and B (500 units/ml of each), was obtained from Lederle Laboratories.

III. PROCEDURE.

Eight dogs were given 8,000 or 10,000 MU/kg of botulinum toxin, type A, by either the iv, ip, or im routes. The approximate iv LD50 for dogs is 3,000 MU/kg, and the im LD50 is 6,000 MU/kg.[2] Venous blood samples (5 ml) were obtained at various times after dosing. These samples were refrigerated until the clots contracted, and the serum was drawn off. The serum was bioassayed for potency of the toxin by ip injection into groups of four or five mice.

Some of the serum samples were serially diluted to obtain partial kills, the amount of dilution depending on the expected concentration of the toxin. The volume of the injected material never exceeded 0.5 ml.

Two types of bioassay were used. One depended upon a quantal response (mortality); the other depended upon a graded response (time to death).

When sufficient partial kills were obtained with a series of serum samples, all of the same volume, from blood drawn over a range of times, the time when this volume contained 1 MU (would kill 50% of the mice) was estimated by plotting the sample times against percent mortality.

When the serum was sufficiently potent to kill most of the mice, time to death was the basis for determining the amount of toxin in the serum. This approach was based on a reference plot of median times to death for various dosages. The reference plot of dose versus time to death (figure 1) was constructed from data supplied by Vocci.[3] The points for plotting this curve are: 1 MU, 5,700 min; 10 MU, 453 min; 100 MU, 318 min; and 1,000 MU, 185 min.

The serum volume of dogs was calculated to be 42 ml/kg, based on a blood weight of 7% of the body weight, a blood specific gravity of 1, and a serum volume equal to 60% of the blood volume.

To test the tolerance of mice to dog serum, 1 ml of serum from a normal dog was injected iv into each of three mice. There were no observable toxic signs; therefore, it was concluded that at least 0.5 ml of serum could be safely injected ip without producing protein shock.

IV. RESULTS.

Table 1 shows the results of range-finding bioassays of toxin in serum from dogs 1, 2, and 3. Since the 21-hr sample from dog 2, which received the toxin im, did not kill any mice, there was less than 1 MU in the 0.5 ml of serum. One-half milliliter of serum from dog 3, which received the toxin iv, drawn at various intervals from 15 min to 21 hr killed all mice. The same dog received 2,500 units of antitoxin iv at 24 hr, and a blood sample taken at 27 hr failed to kill mice or cause any toxic signs.

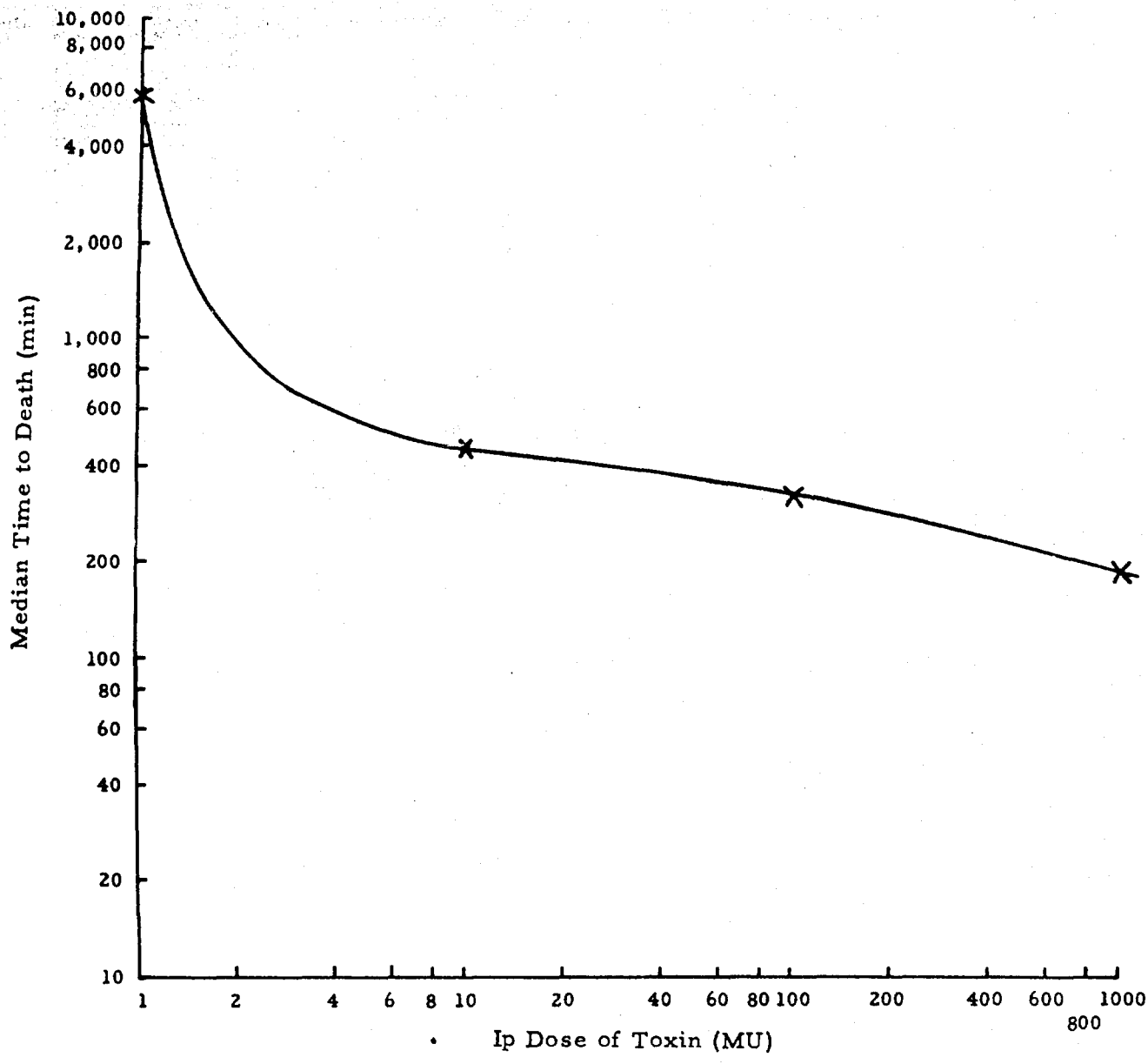

FIGURE 1

MEDIAN TIMES TO DEATH IN MICE AT VARIOUS IP DOSES OF BOTULINUM TOXIN

TABLE 1

RANGE-FINDING BIOASSAY OF BOTULINUM TOXIN IN DOG SERUM AFTER IV AND IM ADMINISTRATION OF 10,000 MU/KG OF TOXIN

(Volume of serum in solution injected in mice was 0.5 ml)

Dogs			Mice	
Animal number	Administration route	Time of blood sampling after toxin	Administration route	Mortality fraction
		hr		
1	Im	5 days	Iv a/	0/3
2	Im	21 b/	Ip	0/5
3	Iv	0.25	Iv	5/5
		1.8	Iv	5/5
		4.2	Ip	4/4
		21	Ip	4/4
		27 b/	Ip	0/4
		1.8 c/	Ip	3/3

a/ Approximate iv LD50 for mice is 3 MU for 25-gm mouse.

b/ Antitoxin administered 24 hr after toxin.

c/ Serum sample allowed to stand 24 hr at room temperature (27°C) before injection into mice.

Table 2 contains data on the bioassay of serum from the blood of dog 4, which received 10,000 MU/kg of toxin iv. The time when a particular volume of serum contained 1 MU was estimated. From the calculated concentration, the percent of injected dose remaining in the blood at this time was estimated (figure 2). At 25.5 hr, only 8% remained. At 63 hr, the amount of toxin in the serum had decreased to 1% of the injected dose.

Table 3 lists the results of the bioassay of serum from dog 5, which received 10,000 MU/kg of toxin im. The median times to death of the mice are listed where they are known. From the reference plot of Lt50 versus ip dose in mice (figure 1), the amount of toxin contained in the serum was read and is shown in table 3. The amount of toxin ranged from <1% at 15 min to a maximum of 11% at 22 hr and then decreased to 1% at 79 hr. At 101 hr there was still 1% of the injected toxin in the serum, and this amount (2.4 to 2.8 MU/ml) was sufficient to kill 2/2 mice. These data are plotted in figure 2.

TABLE 2

BIOASSAY OF BOTULINUM TOXIN IN SERUM OF DOG 4 AT VARIOUS TIMES AFTER IV ADMINISTRATION OF 10,000 MU/KG OF TOXIN

Dog	Mice		Calculations				
			Mice		Dog		
Time of blood sampling after toxin	Volume of serum in ip-injected solution	Mortality fraction	Volume of serum in ip-injected solution	Sample time for 50% kill	Concentration of toxin in serum	Amount of toxin in serum	Amount of injected dose found in serum
hr	ml		ml	hr	MU/ml	MU/kg	%
0.25	0.005	0/5	0.005	0.25	<200	<8,400	<84
			0.005	<0.25	200	8,400	84
0.25	0.05	4/5	0.05	25.5	20	840	8
22.0	0.05	5/5					
29.0	0.05	0/5					
0.25	0.125	5/5	0.125	40	8	336	3
22.0	0.125	5/5					
29.0	0.125	3/3					
46.0	0.125	1/5					
52.5	0.125	0/5					
0.25	0.5	5/5	0.5	63	2	84	1
29.0	0.5	4/5					
46.0	0.5	5/5					
52.5	0.5	4/5					
71.5	0.5	1/4					
79.0	0.5	0/5					

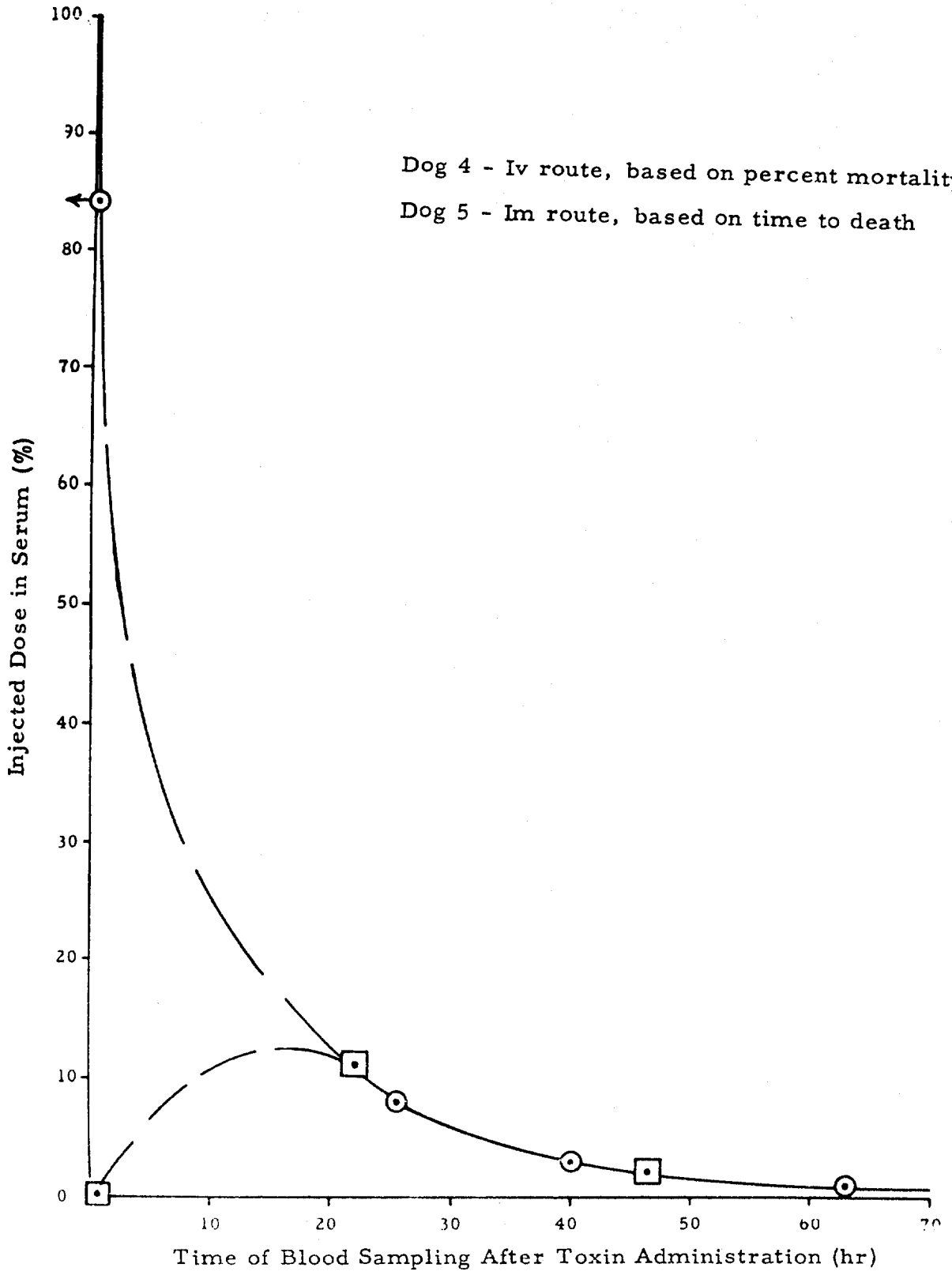

FIGURE 2

AMOUNT OF INJECTED DOSE OF BOTULINUM TOXIN IN DOG SERUM AT VARIOUS TIMES AFTER PARENTERAL ADMINISTRATION (10,000 MU/KG)

TABLE 3

BIOASSAY OF BOTULINUM TOXIN IN SERUM OF DOG 5 AT VARIOUS TIMES AFTER IM ADMINISTRATION OF 10,000 MU/KG OF TOXIN

Dog	Mice				Dog		
Time of blood sampling after toxin	Volume of serum in ip-injected solution	Mortality fraction	Lt50: median time to death after serum	Dose of toxin based on Lt50 versus dose curve	Concentration of toxin in serum	Amount of toxin in serum	Amount of injected dose found in serum
hr	ml		hr	MU	MU/ml	MU/kg	%
0.25	0.5	0/5	—	—	<2.0	<84	<1
22.0	0.5	5/5	7.3	13.0	26.0	1,092	11
29.0	0.5	5/5	<19.8	—	—	—	—
0.5	0.5	5/5	<24.0	—	—	—	—
71.5	0.5	5/5	<23.0	—	—	—	—
79.0	0.5	5/5	23.0 - 45.0	1.6 - 1.2	3.2 - 2.4	134.4 - 100.8	1
101.0	0.5	2/2	30.0 - 45.0	1.4 - 1.2	2.8 - 2.4	117.6 - 100.8	1
46.0	0.25	5/5	44.0	1.2	4.8	201.6	2
52.5	0.125	2/5	—	—	—	—	—
71.5	0.125	0/5	—	—	—	—	—

Table 4 shows the data from the bioassay of botulinum toxin in the serum of dogs 6, 8, and 7, which received 8,000 MU/kg of toxin by the iv, ip, and im routes, respectively. Dosages contained in the serum were determined based on median time to death (table 4). Figure 3 is a plot of these data based on the time to death, except that one point for dog 8 was also calculated from percent mortality. When the toxin was given ip (dog 8), the amount in the serum rose from 0.3% at 2 min to a maximum of 13% at 2.2 hr and then decreased to 4% at 36.3 hr. When the calculation is based on percent mortality, at 2.2 hr 0.05 ml of serum killed 2/4 mice; therefore, the estimated dose in the injected volume was 1 MU, 840 MU/kg, or 10.5% of the injected dose, a value approximately the same as the one calculated on the basis of time to death.

When the to. in was given im (dog 7), the amount found in the serum increased from 5% at 5.7 hr to a maximum of 9% at 12.2 hr and then decreased to 2% at 49.7 hr.

A comparison of the total area under the three curves in figure 3 shows that before 22 hr, the total amount of toxin present in the blood is greatest after iv injection, somewhat less after ip injection, and least after im injection. After 22 hr, the percent of toxin in the serum is the same for the three routes.

Toxic signs did not appear in the dogs until 24 hr after dosing. They appeared approximately in the following order: general weakness and stumbling, dysphagia, aphagia, drooling, various degrees of diarrhea, loss of posterior body support, dehydration and loss of weight, ptosis and lack of blinking, dry and ulcerated cornea, halitosis, thoracic muscular weakness, followed by abdominal breathing and death. All dogs in this test died except dogs 2 and 3, which received antitoxin 24 hr after the toxin. Time of death for dog 6 (iv) was 50 hr, for dog 8 (ip) was 36 hr, and for dog 7 (im) was 97 hr. Time of death for the other dogs was not recorded.

The most characteristic toxic signs in mice after ip administration of dog serum containing toxin are sunken flanks and belly drop (sagging of the abdominal viscera when a mouse is suspended by the tail). The appearance of these two signs is very strong evidence for the diagnosis of botulism.

TABLE 4

BIOASSAY OF BOTULINUM TOXIN IN DOG SERUM AT VARIOUS TIMES AFTER IV, IP, AND IM ADMINISTRATION OF 8,000 MU/KG OF TOXIN

Dogs	Mice					Dogs		
Time of blood sampling after toxin	Volume of serum in ip-injected solution	Mortality fraction		Lt50: median time to death after serum	Dose of toxin based on Lt50 versus dose curve	Concentration of toxin in serum	Amount of toxin in serum	Amount of injected dose found in serum
		1 Day	7 Days					
hr	ml			hr	MU	MU/ml	MU/kg	%
				Dog 6 (Intravenous)				
(3 min)	0.01	1/3	3/3	25.0	1.5	150	6,300	79
0.5	0.01	2/4	4/4	29.0	1.4	140	5,880	74
1.0	0.01	0/4	4/4	34.0	1.3	130	5,460	68
2.3	0.02	2/4	4/4	22.0	1.6	80	3,360	42
5.9	0.05	3/4	3/4	11.5	3.0	60	2,520	32
12.4	0.05	3/4	4/4	22.0	1.6	32	1,344	17
22.5	0.125	4/4	4/4	18.5	1.8	14.4	605	8
36.4	0.5	5/5	5/5	10.8	3.3	6.6	277	3
49.9	0.5	3/5	5/5	19.5	1.7	3.4	143	2
				Dog 8 (Intraperitoneal)				
(2 min)	0.5	0/4	0/4*	—	(0.3)	(0.6)	25	(0.3)
0.4	0.05	0/4	0/4*	—	(0.3)	(6.0)	252	(3.0)
0.9	0.05	0/4	0/4*	—	(0.3)	(6.0)	252	(3.0)
2.2	0.05	0/4	2/4	47.0	1.2	24.0	1,008	13
5.8	0.125	4/4	4/4	11.5	2.9	23.2	974	12
12.3	0.125	4/4	4/4	11.5	2.9	23.2	974	12
22.5	0.125	2/3	3/3	18.4	1.8	14.4	605	8
36.3	0.5	5/5	5/5	10.7	3.4	6.8	286	4
				Dog 7 (Intramuscular)				
3.6	0.5	4/4	4/4	<15.6	>2.1	>4.2	>176	>2
5.7	0.167	3/4	4/4	20.6	1.7	10.2	428	5
12.2	0.167	3/4	3/4	11.3	3.0	18.0	756	9
22.4	0.167	3/4	4/4	12.8	2.5	15.0	630	8
36.2	0.167	1/5	4/5	34.0	1.3	7.8	328	4
49.7	0.5	4/5	4/5	22.4	1.6	3.2	134	2

* For plotting 0% mortality by Berkson method [Berkson, J. A Statistically Precise and Relatively Simple Method of Estimating the Bio-Assay With Quantal Response Based on the Logistic Function. J. Am. Statist. Assoc. 48, 565-599 (1953)], mortality fraction of 0/4 is estimated to be equivalent to mortality of 1/8 or 12.5%, which is expected mortality in mice at dose of 0.3 MJ.

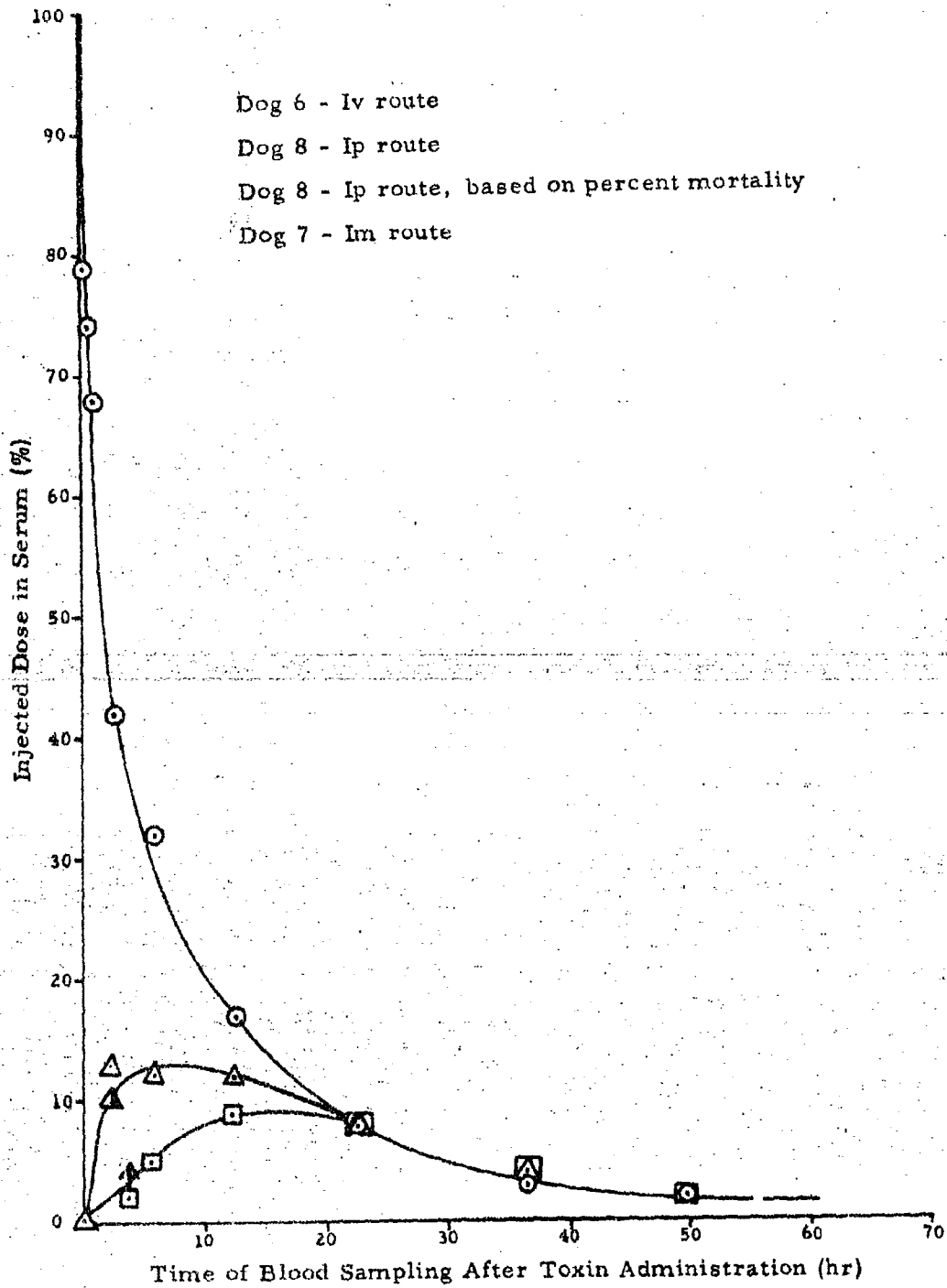

FIGURE 3

AMOUNT OF INJECTED DOSE OF BOTULINUM TOXIN IN DOG SERUM AT VARIOUS TIMES AFTER PARENTERAL ADMINISTRATION (8,000 MU/KG)

V. DISCUSSION.

The ip and im curves in figure 2 represent the net result of toxin entering and leaving the blood. The peak concentration represents the point of equilibrium, when the amount of toxin entering the blood equals the amount leaving the blood. Toxin entering the bloodstream from the tissues leaves it at the same rate as the iv-injected toxin. As expected, toxin injected by the im route is slowly absorbed into the bloodstream, and small amounts of it may remain in the blood for as long as 4 days. The toxic signs occurred in the dog receiving the im dose somewhat later (36 to 48 hr after the toxin) than in the dogs receiving the ip or iv doses.

When the toxin is ingested, the process of absorption is different from that when the toxin is given by the ip and im routes. The lethal oral dose[4] (for dogs it is 1,200,000 MU) is many times greater than lethal parenteral dose. Only a very small portion of an oral dose is absorbed, and this occurs over periods of hours or at least as long as the toxin is present in the alimentary canal. The serum level at any given time is not likely high. Nevertheless, the general pattern for changes in the concentration in serum after ingestion of toxin may be similar to the patterns for ip and im injection.

In the author's work on mice[5] that received 80 MU of toxin per mouse iv, antitoxin administered 1 hr after the toxin saved all the mice, but when antitoxin was injected 2 hr after the toxin, all the mice died. In the dog, approximately 32% of the iv dose was undetectable at 1 hr and presumably had left the bloodstream. If this rate of disappearance occurs in the mouse, then it can be assumed that 80 MU $\times$ 0.32 or 25.6 MU of toxin had disappeared from the blood after 1 hr. This is approximately 9 iv LD50's, since 1 iv LD50 = 3 MU. Yet, antitoxin administered iv saved all the mice. This implies that not all the toxin that left the blood was immediately bound at the site of action. Also, it may be that a portion of the toxin had either been inactivated by natural processes or was still en route to the site of action. After a certain time, antitoxin therapy has little value. This is believed to indicate that the toxin has then become fixed at the receptor site. Dog 3, which received antitoxin 27 hr after 10,000 MU/kg of toxin, presumably had only approximately 8% of this dose remaining in his blood (based on dog 4) when the antitoxin was administered. If all the toxin that had left the blood were fixed at receptor sites, then the dog probably would not have survived after the antitoxin or, if the animal did survive, it at least would have shown toxic signs. The time factor for the presence of toxin in the blood may be of greater importance for toxic effects than is its actual concentration. By the three routes studied, the blood levels are nearly identical after 22 hr and remain identical, although decreasing, for hours thereafter.

The mouse bioassay, as used in this experiment, is applicable as a diagnostic laboratory procedure for botulism in humans. By this procedure it should be possible to determine the presence of botulinum toxin in a specimen, its concentration, and its type. The type of toxin may be determined by administration of each of the various specific antitoxins, types A, B, C, D, and E, to groups of mice poisoned with the specimen containing the toxin in question. The absence of toxic signs in any one of the groups treated identifies the type. All other groups, including the untreated control, should show toxic signs.

VI. CONCLUSIONS.

It was concluded that:

1. When botulinum toxin is administered ip and im to dogs, peak serum levels are reached at 12 hr (ip = 12%; im = 9%). At 22 hr, blood levels are identical (8.5%) by the iv, im, and ip routes and remain identical, although decreasing, for many hours thereafter.

2. Botulinum toxin administered iv, ip, and im in doses of 10,000 MU/kg to dogs can be detected in the serum by bioassay in mice for as long as 2 to 4 days after injection.

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5. Crook, J. W., Cresthull, P., and Oberst, F. W. The Effectiveness of Botulism Antitoxin and Drugs Against Experimental Botulism in Mice. (In process.)

ABSTRACT

1. ORIGINATING ACTIVITY - U. S. Army Edgewood Arsenal Chemical Research and Development Laboratories, Edgewood Arsenal, Maryland - Toxicology Division

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2b. GROUP -

3. REPORT TITLE - CHANGES IN CONCENTRATION OF BOTULINUM TOXIN IN DOG SERUM AFTER PARENTERAL ADMINISTRATION (U)

4. DESCRIPTIVE NOTES - The work was started in February 1963 and completed in June 1963.

5. AUTHORS - House, Michael J., Cresthull, Paul, Crook, James W., Oberst, Fred W.

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10. AVAILABILITY/LIMITATION NOTICES - Qualified requesters may obtain copies of this report from Defense Documentation Center, ATTN: TISIA-2, Cameron Station, Alexandria, Virginia

11. SUPPLEMENTARY NOTES - Medical defense and aspects of chemical agents.

12. SPONSORING ACTIVITY -

13. ABSTRACT

(U) This experiment was planned to determine the amount of free botulinum toxin, Type A, in blood of dogs at various times after parenteral administration. The toxin was administered to the test subjects in doses of 8,000 and 10,000 MU*/kg by the intravenous, intraperitoneal, and intramuscular routes. Blood samples were taken at various times after injection, and the concentration of toxin in the serum was determined by a bioassay in mice using the mortality fraction or the times of death. The percent of injected dose found in the serum was plotted against sampling time to show the extent of transfer of toxin into the blood stream from the intraperitoneal and the intramuscular routes and its disappearance from the blood. Botulinum toxin administered intravenously, intraperitoneally, and intramuscularly in doses of 10,000 MU/kg to dogs can be detected in the serum by bioassay in mice as long as 2 to 4 days after injection.

14. KEYWORDS

Botulinum toxin
Antitoxin
Intraperitoneal
Intramuscular
Intravenous
Serum
Concentration
Dogs
Blood
Bioassay
Sampling
Toxin
Mortality
Mice

* MU - the mouse intraperitoneal LD50.